Prognostic impact of CD8-positive tumour-infiltrating lymphocytes and PD-L1 expression in salivary gland cancer

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Abstract: Objectives: Aim of the study was to evaluate the prognostic impact of CD8-positive (CD8+) tumour-infiltrating lymphocytes (TILs) and PD-L1 expression on the outcome of patients with malignant salivary gland neoplasms. Materials and methods: Formalin-fixed, paraffin-embedded tissue samples and clinicopathological data from patients treated for salivary gland carcinoma in a head and neck cancer centre were retrospectively retrieved. Immunohistochemical staining was applied on sections of 84 specimens of 12 different histological subtypes. Both CD8 and PD-L1 expression were rated by semi-automated cell counts by a digital image analysis programme. Survival analyses were performed by the log-rank test on the univariate level, and the Cox model was applied on the multivariate level. Associations between immunological markers and clinicopathological variables were estimated by the Pearson chi-squared test. Additionally, PD-1 was estimated as an exhaustion marker of CD8+ TILs. Results: Patients exceeding a tumour proportion score 5% regarding PD-L1 expression demonstrated a significantly decreased survival, as did individuals with an overall high CD8+ cell density. Particularly, high CD8+ cell counts in the invasive front of the respective tumour tissue significantly coincided with a poor outcome. Also, high numbers of CD8+ TILs significantly matched with a high quantity of PD-1+ TILs. Conclusion: CD8+ TILs abundance in the peritumoural microenvironment correlates with impaired outcome of patients with salivary gland carcinoma. The simultaneous negative prognostic impact of PD-L1 expression and presence of PD-1+ TILs advocates an immune checkpoint-controlled mechanism of CD8+ TILs exhaustion for these tumours and paves the way for future treatment strategies.

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ABSTRACT

Objectives: Aim of the study was to evaluate the prognostic impact of CD8-positive ($CD8^+$) tumour-infiltrating lymphocytes (TILs) and PD-L1 expression on the outcome of patients with malignant salivary gland neoplasms.
Materials and methods: Formalin-fixed, paraffin-embedded tissue samples and clinicopathological data from patients treated for salivary gland carcinoma in a head and neck cancer centre were retrospectively retrieved. Immunohistochemical staining was applied on sections of 84 specimens of 12 different histological subtypes. Both CD8 and PD-L1 expression were rated by semi-automated cell counts by a digital image analysis programme. Survival analyses were performed by the log-rank test on the univariate level, and the Cox model was applied on the multivariate level. Associations between immunological markers and clinicopathological variables were estimated by the Pearson chi-squared test. Additionally, PD-1 was estimated as an exhaustion marker of $CD8^+$ TILs.
Results: Patients exceeding a tumour proportion score $\geq 5\%$ regarding PD-L1 expression demonstrated a significantly decreased survival, as did individuals with an overall high $CD8^+$ cell density. Particularly, high $CD8^+$ cell counts in the invasive front of the respective tumour tissue significantly coincided with a poor outcome. Also, high numbers of $CD8^+$ TILs significantly matched with a high quantity of PD-1^+ TILs.
Conclusion: $CD8^+$ TILs abundance in the peritumoural microenvironment correlates with impaired outcome of patients with salivary gland carcinoma. The simultaneous negative prognostic impact of PD-L1 expression and presence of PD-1^+ TILs advocates an immune checkpoint-controlled mechanism of $CD8^+$ TILs exhaustion for these tumours and paves the way for future treatment strategies.

Introduction

Malignant salivary gland neoplasms encompass a rare tumour entity covering only three percent of all head and neck cancers [1]. Moreover, a diverse range of twenty histological subtypes and their incoherent clinical behaviour pose a challenging situation upon treatment rationale [2,3]. Although there is lack of evidence-based studies providing structured recommendations, surgery represents the favoured option in therapy algorithm [1,2,4–6], followed by several modalities of radiotherapy [7,8].

In recent years, an interest has developed towards personalised therapy and treatment de-escalation in salivary gland cancer. Molecular aberrations already have been reported as potential therapeutic targets [9], beside the attempt to implement immunomodulatory concepts [10]. Immune checkpoints like the programmed death receptor-1 (PD-1) and one of its corresponding ligands (PD-L1) are gaining scientific interest, as they have proven to play a pivotal role in the outcome of several other malignancies [9,11,12]. While PD-1 is expressed on activated T-cells, PD-L1 is found on the surface of the cancer cell. Formation of their receptor-ligand-complex results in T-cell exhaustion and

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suppression of cytotoxic cluster of differentiation 8-positive ($CD8^+$) tumour-infiltrating lymphocytes (TILs), with both mechanisms enabling tumour immune escape. On the contrary, immune checkpoint blockade and an effective response of the tumour microenvironment have been identified to coincide with the presence of $CD8^+$ cells [13], and in consequence, with a survival benefit [14]. Accordingly, PD-L1 as well as $CD8^+$ TILs propose useful biomarkers in the profiling of subjects, who might profit from immunotherapeutic strategies [13].

Thus far, a few studies have examined the prognostic role of the PD-1/PD-L1 axis for salivary gland malignancies [15–26], whereas the impact of $CD8^+$ cells remains uncertain [16,21]. A difficulty all aforementioned investigations have in common, is that sparse attention has been paid to the interaction between both immunological markers, so that the therapeutic merit of immunomodulatory agents in those tumours might be unfavourable, due to an improbable response [13].

Hence, the aim of our single centre study was to retrospectively evaluate the prognostic impact of both $CD8^+$ TILs and PD-L1 expression on the outcome of a patient cohort with head and neck salivary gland cancer.

Materials and methods

Study population and treatment

The trial was reviewed and approved by the local university ethics committee based on the Declaration of Helsinki in its actual, revised form. Patients with malignant salivary gland tumours treated by surgery and/or radiotherapy were eligible for the study. Neoplasms originating from the three paired major salivary glands, the parotid, submandibular and sublingual ones, and the minor salivary glands of the oral cavity were considered. Data were collected from the Department of Radiotherapy and Oncology, the Department of Oral, Maxillofacial and Facial Plastic Surgery and the Department of Otorhinolaryngology of a university cancer centre.

Electronic and paper-based charts were screened and a population of 127 individuals could be identified (Table 1). All patients received unimodal or multimodal treatment of primary or recurrent salivary gland cancer between 2002 and 2017. Regarding primary therapy, a total of 121 individuals had surgery, of whom 92 underwent neck dissection. Irradiation was performed in 62 patients as 3D-conformal or intensity-modulated radiotherapy in a post-operative or definitive manner. Systemic treatment was administered in 19 patients, mostly platinum-based in the post-operative setting as an individual therapeutic decision.

Histopathological analysis

Formalin-fixed, paraffin-embedded (FFPE) tissue samples of 84 patients were available. All specimens were re-evaluated by an experienced pathologist and histopathological diagnosis was confirmed based on the latest WHO Classification of Head and Neck Tumours [3]. A pool of 12 different histopathological subtypes could be included (Supplementary Table S1). In case of squamous cell carcinoma (SCC), due to its rare appearance as primary salivary gland malignancy, the final diagnosis was clarified after exclusion of any other primary tumour site by clinical (including dermatological) and radiographical examination.

The respective samples were grouped into a cohort with either aggressive or non-aggressive characteristic based upon their histopathological subtype. Acinic cell carcinoma (ACC) as well as low- and intermediate-grade mucoepidermoid carcinoma (MEC G1-G2) were classified as non-aggressive [3,27]. Adenoid cystic carcinoma (AdCC), adenocarcinoma – not otherwise specified (AdenoCa), salivary duct carcinoma (SDC), SCC and high-grade mucoepidermoid carcinoma (MEC G3) were stated as aggressive [3,28,29]. Any other remaining low-grade (G1) histopathological subtypes were defined as non-aggressive, and similarly, any other remaining high-grade (G3) entities as aggressive [3].

Table 1
Clinicopathological characteristics.

	n = 127 (%)
Gender	
Male	69 (54.3)
Female	58 (45.7)
Age (range, 9–93 years)	
< Median (61 years)	62 (48.8)
≥Median	65 (51.2)
Tumour site	
Parotid gland	92 (72.4)
Submandibular gland	10 (7.9)
Sublingual gland	2 (1.6)
Minor salivary glands	23 (18.1)
T-stage	
T1	44 (34.6)
T2	17 (13.4)
T3	31 (24.4)
T4	25 (19.7)
Missing information	10 (7.9)
N-stage	
N0	84 (66.1)
N1	18 (14.2)
N2	24 (18.9)
N3	1 (0.8)
M-stage	
M0	108 (85.0)
M1	4 (3.1)
MX	15 (11.8)
Grade	
G1	23 (18.1)
G2	68 (63.5)
G3	36 (28.3)
Resection margins[a]	
R0	77 (63.6)
R1	37 (30.6)
R2	7 (5.8)

[a] Total n = 121 due to six non-operated patients.

Immunohistochemical analysis

Sections of 5 μm thickness were deparaffinised in xylene and rehydrated in graded alcohol. Heat-induced epitope retrieval was performed in a water bath at 97 °C with a Tris-EDTA buffered solution at pH 9.0 (Target Retrieval Solution; Dako, Glostrup, Denmark). Endogenous peroxidase activity was inhibited with hydrogen peroxide (Peroxidase-Blocking Reagent; Dako). Primary antibodies were applied directed against either CD8 (monoclonal mouse, clone C8/144B; Dako) or PD-L1 (monoclonal rabbit, clone ZR3; Zeta Corporation, Arcadia, CA, USA). The antigen-antibody-complexes were visualised with dextran-polymer conjugated horseradish-peroxidase (/HRP; Dako) and 3,3′-diaminobenzidine (DAB + Chromogen; Dako). All specimens were counterstained with haematoxylin (Gill No. 3; Sigma-Aldrich, Saint Louis, MO, USA). Positive control slides prepared with known samples of oropharyngeal SCC and negative control slides without primary antibodies were included into each staining cycle.

Images were acquired with the AxioScan Z.1 slide scanner and matching ZEN software (both Carl Zeiss Microscopy, Jena, Germany). The number of $CD8^+$ TILs and PD-L1 expressing cells was evaluated for each sample using the open source programme QuPath (QuPath, v0.1.2, Belfast, Northern Ireland, UK) [30]. The previously identified tumorous areas were contoured and the abundance of both CD8 and PD-L1 positive cells per mm^2 tissue was automatically quantified within the delineated parts.

The number of $CD8^+$ TILs was assessed in three distinct tissue compartments, distinguished as intratumoural area, stroma, and

Table 2
Univariate and multivariate analysis on the impact of prognostic factors with respect to OS, LPFS, DMFS and PFS.

	Univariate analysis	Schoenfeld test	Multivariate analysis		
	p-value	p-value	HR	95% CI	p-value
OS (n = 28 events included into MVA)		0.433 (global)			
Gender (male vs. female)	0.188				
Age (< median of 61 years vs. ≥ median)	< **0.001**				
T-stage (T1-T2 vs. T3-T4)	< **0.001**	0.106	5.500	1.831–16.527	**0.002**
N-stage (N0 vs. N1-N3)	< **0.001**				
Subtype characteristic (non-aggressive vs. aggressive)	**0.001**	0.985	1.802	0.438–7.404	0.414
$CD8^+$ total (low vs. high)	**0.046**				
$CD8^+$ intratumoural (low vs. high)	0.427				
$CD8^+$ stroma (low vs. high)	0.882				
$CD8^+$ invasive front (low vs. high)	**0.010**	0.312	4.076	1.609–10.324	**0.003**
PD-L1 (TPS < 5% vs. TPS ≥ 5%)	**0.002**	0.586	1.053	0.424–2.621	0.911
$PD\text{-}1^+$ (low vs. high)	0.735				
LPFS (n = 33 events included into MVA)		0.480 (global)			
Gender (male vs. female)	0.194				
Age (< median of 61 years vs. ≥ median)	< **0.001**				
T-stage (T1-T2 vs. T3-T4)	< **0.001**	0.370	6.140	2.186–17.248	**0.001**
N-stage (N0 vs. N1-N3)	< **0.001**				
Subtype characteristic (non-aggressive vs. aggressive)	**0.006**	0.450	1.102	0.339–3.582	0.871
$CD8^+$ total (low vs. high)	0.214				
$CD8^+$ intratumoural (low vs. high)	0.432				
$CD8^+$ stroma (low vs. high)	0.877				
$CD8^+$ invasive front (low vs. high)	**0.030**	0.790	3.200	1.394–7.348	**0.006**
PD-L1 (TPS < 5% vs. TPS ≥ 5%)	**0.008**	0.993	1.001	0.420–2.383	0.999
$PD\text{-}1^+$ (low vs. high)	0.946				
DMFS (n = 31 events included into MVA)		0.935 (global)			
Gender (male vs. female)	0.096				
Age (< median of 61 years vs. ≥ median)	< **0.001**				
T-stage (T1-T2 vs. T3-T4)	< **0.001**	0.576	5.352	1.942–14.751	**0.001**
N-stage (N0 vs. N1-N3)	< **0.001**				
Subtype characteristic (non-aggressive vs. aggressive)	< **0.001**	0.794	2.350	0.628–8.789	0.204
$CD8^+$ total (low vs. high)	0.051				
$CD8^+$ intratumoural (low vs. high)	0.309				
$CD8^+$ stroma (low vs. high)	0.503				
$CD8^+$ invasive front (low vs. high)	**0.047**	0.451	3.929	1.602–9.632	**0.003**
PD-L1 (TPS < 5% vs. TPS ≥ 5%)	**0.011**	0.704	0.785	0.311–1.976	0.607
$PD\text{-}1^+$ (low vs. high)	0.676				
PFS (n = 35 events included into MVA)		0.827 (global)			
Gender (male vs. female)	0.108				
Age (< median of 61 years vs. ≥ median)	< **0.001**				
T-stage (T1-T2 vs. T3-T4)	< **0.001**	0.709	5.900	2.218–15.695	< **0.001**
N-stage (N0 vs. N1-N3)	< **0.001**				
Subtype characteristic (non-aggressive vs. aggressive)	**0.004**	0.467	1.425	0.463–4.389	0.537
$CD8^+$ total (low vs. high)	0.080				
$CD8^+$ intratumoural (low vs. high)	0.209				
$CD8^+$ stroma (low vs. high)	0.531				
$CD8^+$ invasive front (low vs. high)	**0.033**	0.860	3.943	1.684–9.232	**0.002**
PD-L1 (TPS < 5% vs. TPS ≥ 5%)	**0.016**	0.978	0.768	0.314–1.878	0.563
$PD\text{-}1^+$ (low vs. high)	0.750				

Age as a multi-influenced variable was excluded a priori from MVA. Additionally, due to the multi-collinear character of the variables nodal stage and tumour stage ($p < 0.001$, correlation given by two-sided Spearman-Rho test), only the latter one was considered. $CD8^+$ total as variable in OS was not included in the respective MVA.

Abbreviations: MVA, multivariate analysis; OS, overall survival; LPFS, local progression-free survival; DMFS, distant metastases-free survival; PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; TPS, tumour proportion score.

invasive front. A total $CD8^+$ cell score per specimen was defined by summarising the three compartmental values. Based on the respective median, the study population was categorised into a group with either low or high number of $CD8^+$ TILs [31]. The prevalence of PD-L1 expression was amounted in accordance to the tumour proportion score (TPS) as introduced by Garon et al. [32], and a level of ≥5% was chosen as threshold to dichotomise the patient cohort [19].

To assess the functionality of $CD8^+$ TILs and to detect any signs of exhaustion [33], the status of PD-1-positive ($PD\text{-}1^+$) TILs was additionally analysed. Primary antibodies against PD-1 (monoclonal rabbit, clone EP239; DCS, Hamburg, Germany) were applied following the immunohistochemical protocol as depicted above, and the cell density was estimated using QuPath. As the expression and staining intensity of $PD\text{-}1^+$ cells was weak, the number of $PD\text{-}1^+$ TILs was measured on a total level instead of in three separate tissue compartments. Given by the median, the patient population was divided into a group with low or high abundance of $PD\text{-}1^+$ TILs [11].

Statistical analysis

Statistical procedures were calculated using R (The R Foundation for Statistical Computing, v3.5.0, Vienna, Austria) and SPSS (IBM SPSS Statistics, v24.0, Armonk, NY, USA).

Survival analyses were performed with respect to overall survival (OS), local progression-free survival (LPFS), distant metastases-free survival (DMFS) and progression-free survival (PFS). The date of diagnosis was defined as start of each interval. The endpoint of interest for OS was death from any cause, for LPFS any sign of locoregional

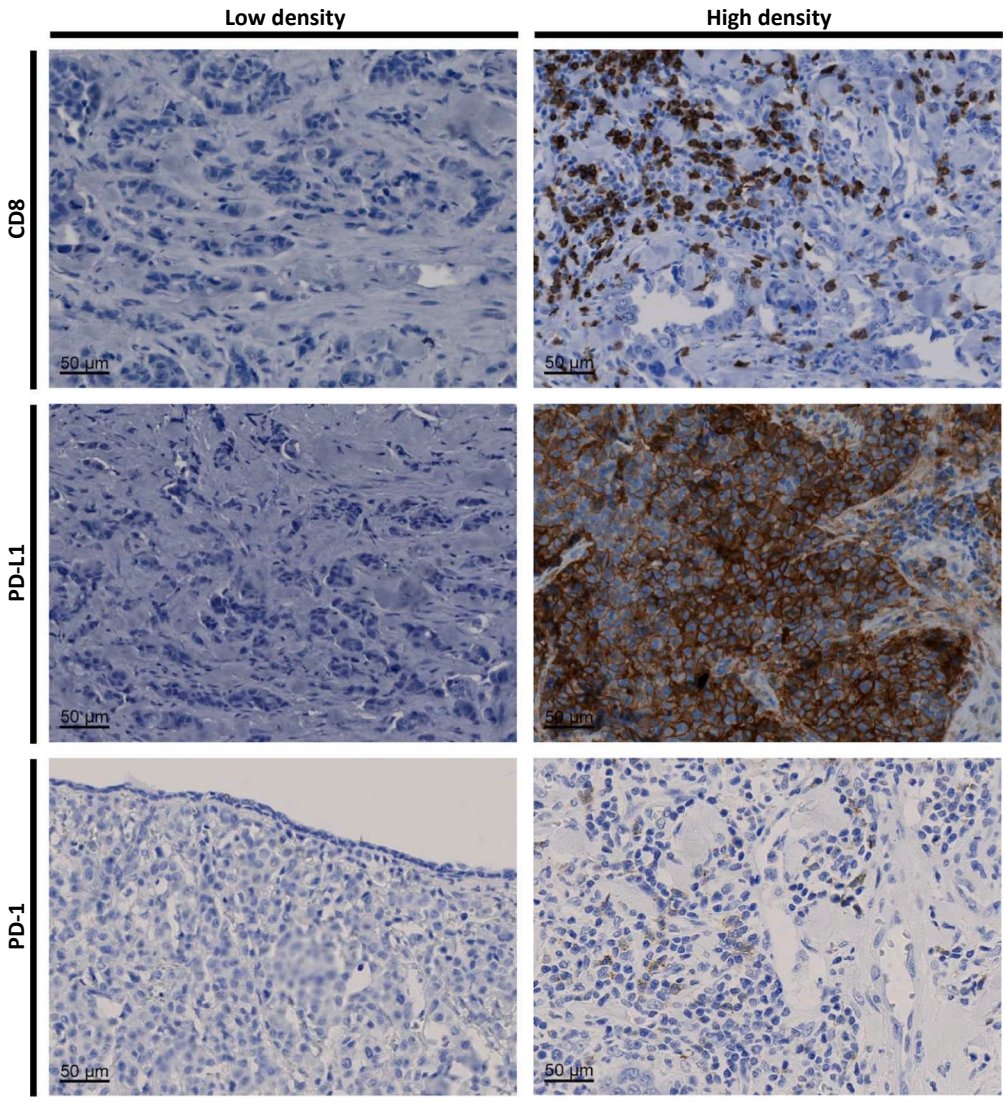

Fig. 1. Representative samples of immunohistochemical stains for CD8, PD-L1 and PD-1 in the most frequent diagnosed histopathological subtype (AdenoCa) in the examined cohort. CD8 staining represents cytotoxic T-cells intratumoural with low and high density based on the median value. PD-L1 staining shows positive cells defined as complete membrane staining intratumoural and staining in peritumoural immune cell infiltrate. A TPS $<$ 5% (low density) and TPS $\geq$ 5% (high density) are presented. PD-1 shows immune cells intratumoural. Low and high density are defined by the median value. Magnification, 200×; scale bar, 50 µm. Abbreviations: AdenoCa, adenocarcinoma – not otherwise specified; TPS, tumour proportion score.

tumour progress or death from any cause, for DMFS any sign of metastatic occurrence or death from any cause and for PFS either any sign of locoregional tumour progress, metastatic occurrence or death from any cause. Patients who were alive and/or without tumour recurrence were censored for the respective event at the last contact. Additionally, the clinical follow-up was considered with the last appointment as endpoint of interest. Univariate analyses were calculated by the log-rank test and survival curves were visualised by the Kaplan-Meier method. Multivariate analyses were based upon the Cox model. The assumption of proportional hazards was verified by assessment of the scaled Schoenfeld residuals on both the individual and global level. To avoid overfitting [34], we strived for a minimum of 10 events per variable (EPV) in each multivariate analysis: Age as a multi-influenced variable was excluded a priori from any Cox model. Due to the multi-collinear character of the variables nodal stage and tumour stage ($p < 0.001$, correlation given by two-sided Spearman-Rho test), only the latter one was considered. Still, we needed to adjust the respective multivariate analyses based on recommendations by Vittinghoff & McCulloch, who indicated statistical problems as uncommon even in case of a model with 5–9 EPV when compared to a model with 10–16 EPV [35].

Association between categorical variables was evaluated by the Pearson chi-squared test.

The level of significance was set at $p \leq 0.05$ for all statistical procedures.

Results

Clinicopathological characteristics and survival

In reference to our grading system, 82 samples (64.6%) with aggressive subtype characteristic were diagnosed on patient level corresponding to 61 retrieved samples (72.6%) on specimen level, whereas 37 samples (29.1%) with non-aggressive subtype characteristic were documented on patient level and 18 samples (21.4%) on specimen level. Eight (6.3%, patient level) and respectively 5 samples (6.0%, specimen level) remained non-classified.

The median clinical follow-up was 30 months (range, 0–421 months) and the median OS was 55 months (range, 0–443 months). At the time of reporting, 88 patients (69.3%) were known to be alive, while 39 patients (30.7%) were deceased. Locoregional failure occurred in 22 patients (17.3%) at a median LPFS of 50 months (range, 0–257 months). Also, 22 patients were diagnosed with respectively multilocal distant metastases at a median DMFS of 50 months (range, 0–443 months). Overall, the median PFS was 47 months (range, 0–257 months). OS and PFS rates reached 91.3% and 82.7% at 12 months, 86.6% and 78.0% at 24 months, 77.2% and 68.5% at 60 months, 73.2% and 65.4% at 120 months, and 70.9% and 62.2% at 180 months, respectively.

In univariate analysis a significantly improved OS was observed in association with younger age ($p < 0.001$), lower tumour stage

(p < 0.001), the absence of nodal involvement (p < 0.001), and a non-aggressive subtype characteristic (p = 0.001). In addition, these parameters were significantly associated with a favourable LPFS, DMFS and PFS. In multivariate analyses, tumour stage was confirmed as independent prognostic variable (Table 2).

Immunological markers and survival

The patient cohort was split at the median value into a group with low (< 616 cells/mm^2) and high (≥616 cells/mm^2) density of CD8$^+$ TILs. Further, the study population was dichotomised concerning spatial distribution including the intratumoural compartment (low density, < 75 cells/mm^2 vs. high density, ≥75 cells/mm^2), stroma (low density, < 62 cells/mm^2 vs. high density, ≥62 cells/mm^2), and invasive front (low density, < 315 cells/mm^2 vs. high density, ≥315 cells/mm^2). Equally, the individuals were categorised into a pool with low (TPS < 5%) and high (TPS ≥ 5%) PD-L1 prevalence in the tumour cells.

Representative samples of salivary gland carcinoma are depicted in Fig. 1 (AdenoCa), and Supplementary Figs. S1 and S2 demonstrating a particularly non-aggressive (ACC) vs. aggressive (SDC) histopathological subtype.

As given by univariate analysis, patients with a low PD-L1 prevalence revealed a significantly superior OS (TPS < 5% vs. TPS ≥ 5%, mean 199 vs. 56 months; p = 0.002), LPFS (TPS < 5% vs. TPS ≥ 5%, mean 128 vs. 54 months; p = 0.008), DMFS (TPS < 5% vs. TPS ≥ 5%, mean 229 vs. 52 months; p = 0.011) and PFS (TPS < 5% vs. TPS ≥ 5%, mean 123 vs. 52 months; p = 0.016) (Table 2, Fig. 2). Notably, patients with a high total CD8$^+$ cell density, were prone to a poor OS (low vs. high density, mean 221 vs. 107 months; p = 0.046). Although lacking significances concerning LPFS, DMFS and PFS, a trend towards a worse outcome in individuals with a high total amount of CD8$^+$ TILs was evident (Table 2, Fig. 3). Particularly, patients with a high CD8$^+$ cell score in the invasive front were susceptible towards a significantly decreased OS (low vs. high density, mean 229 vs. 101 months; p = 0.010), LPFS (low vs. high density, 142 vs. 91 months; p = 0.030), DMFS (low vs. high density, 284 vs. 101 months; p = 0.047), and PFS (low vs. high density, 137 vs. 89 months; p = 0.033) (Table 2, Supplementary Fig. S3), whereas the amount of CD8$^+$ cells in the intratumoural compartment and stroma displayed an indifferent impact on survival (Table 2, Supplementary Figs. S4 and S5). Regarding multivariate analysis, the CD8$^+$ cell score in the invasive front was confirmed as independent prognostic factor (Table 2).

Patients with a CD8$^{+\text{high}}$/PD-L1$^{\text{high}}$ expression pattern were assigned with the worst prognosis, in contrast to individuals with a CD8$^{+\text{high}}$/PD-L1$^{\text{low}}$ and CD8$^{+\text{low}}$/PD-L1$^{\text{low}}$ cluster, respectively. The presented results significantly affected OS (p = 0.004), LPFS (p = 0.027), DMFS (p = 0.023) and PFS (p = 0.036) as illustrated in Fig. 4. Due to just one monitored event, the presumed beneficial impact of a CD8$^{+\text{low}}$/PD-L1$^{\text{high}}$ factor should be only considered for descriptive reasons.

Association of clinicopathological characteristics and immunological markers

A high expression of CD8$^+$ TILs in total and in each of the three examined compartments was significantly associated with higher age. Additionally, a high density of CD8$^+$ cells in total significantly coincided with nodal involvement. In regard to an association between the histopathological subtype and thus the five most frequently diagnosed entities in the present study cohort, ACC, MEC, AdCC, AdenoCa and SCC, and the amount of CD8$^+$ TILs, significant results were obtained on the total and on the intratumoural level, and in the invasive front. Whilst MEC and AdenoCa expressed indifferent behaviour, a high CD8$^+$ cell expression coincided with ACC and SCC, and a low CD8$^+$ cell expression with AdCC. Tumours with an aggressive subtype characteristic were found to significantly coincide with a low CD8$^+$ cell density in the invasive front (Table 3).

With respect to PD-L1, significant results were reported concerning the variables higher age, which was associated with a TPS ≥ 5%, nodal

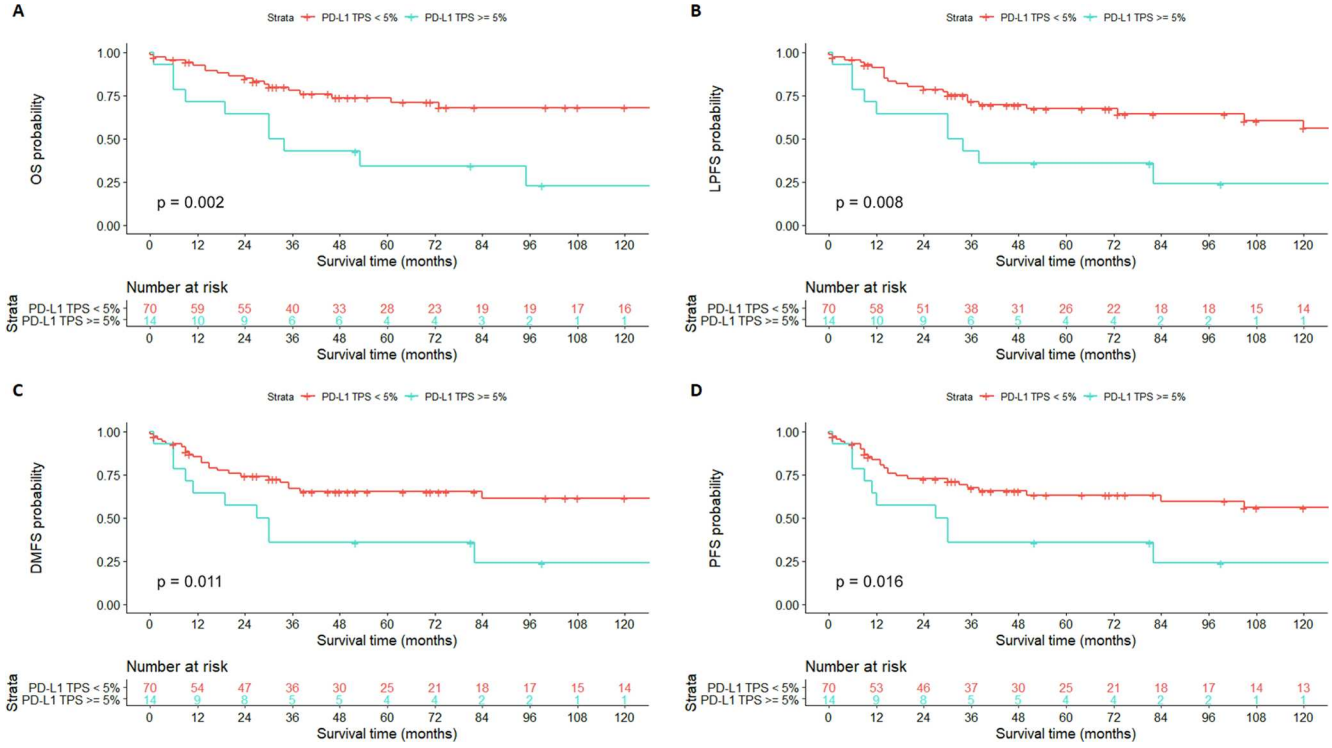

Fig. 2. Prognostic impact of PD-L1 expression on OS (overall survival), LPFS (local progression-free survival), DMFS (distant metastases-free survival) and PFS (progression-free survival).

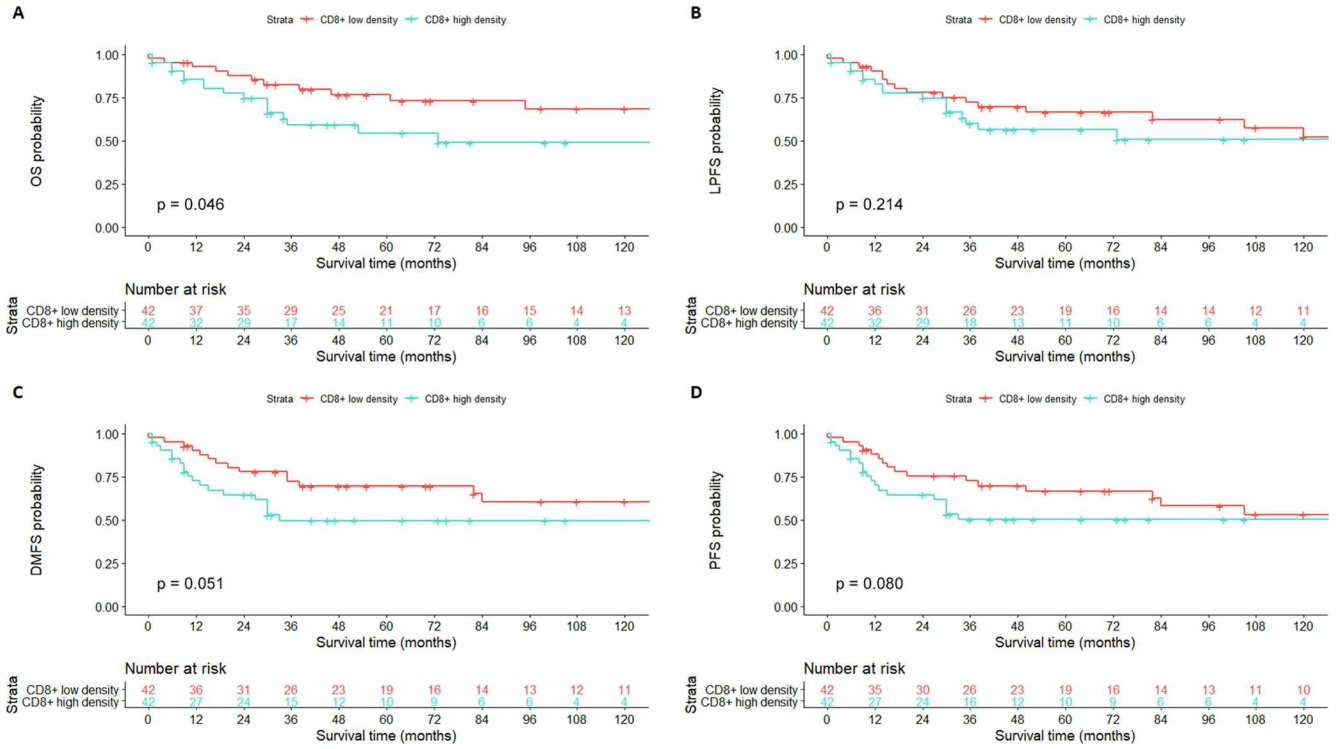

Fig. 3. Prognostic impact of CD8$^+$ tumour-infiltrating lymphocytes on OS (overall survival), LPFS (local progression-free survival), DMFS (distant metastases-free survival) and PFS (progression-free survival).

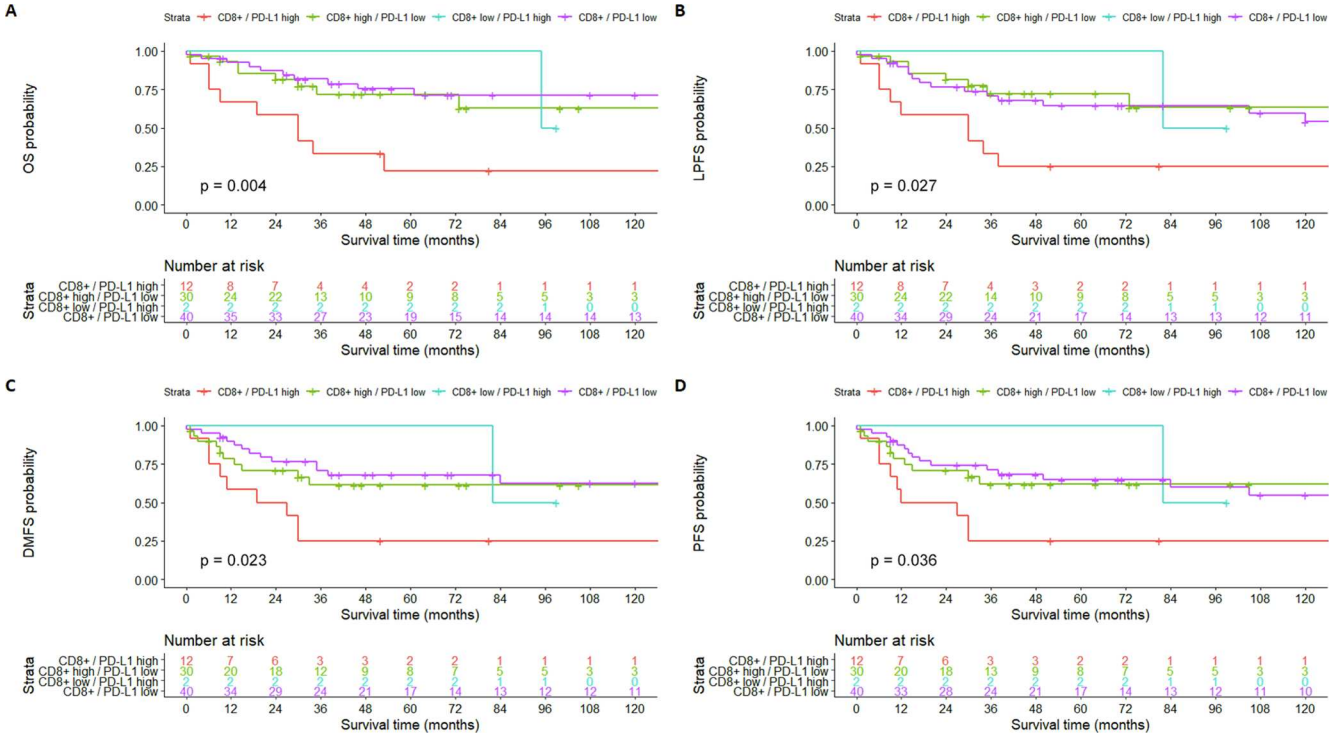

Fig. 4. Prognostic impact of CD8$^+$ tumour-infiltrating lymphocytes and PD-L1 as combined variables on OS (overall survival), LPFS (local progression-free survival), DMFS (distant metastases free) and PFS (progression-free survival).

involvement and histological subtype. The investigated samples of AdCC lacked any PD-L1 expression ≥ 5% at all, in comparison to ACC, MEC, AdenoCa and SCC (Table 3).

Status of PD-1$^+$ tumour-infiltrating lymphocytes

Based on the median value, the study population was divided into a group with low (< 32 cells/mm^2) and high (≥ 32 cells/mm^2) density of PD-1$^+$ TILs. In the Pearson chi-squared test a significant association could be shown between a high density of both CD8$^+$ and PD-1$^+$ TILs

Table 3
Association of clinicopathological characteristics and immunological markers.

	CD8$^+$ total			CD8$^+$ intratumoural			CD8$^+$ stroma		
	Low **n = 42 (%)**	High **n = 42 (%)**	p-value[a]	Low **n = 42 (%)**	High **n = 42 (%)**	p-value[a]	Low **n = 42 (%)**	High **n = 42 (%)**	p-value[a]
Gender									
Male	26 (61.9)	25 (59.5)	0.823	23 (54.8)	28 (66.7)	0.264	26 (61.9)	25 (59.5)	0.823
Female	16 (38.1)	17 (40.5)		19 (45.2)	14 (33.3)		16 (38.1)	17 (40.5)	
Age (range, 9–93 years)									
< Median (61 years)	25 (59.5)	14 (33.3)	**0.016**	24 (57.1)	15 (35.7)	**0.049**	25 (59.5)	14 (33.3)	**0.016**
≥Median	17 (40.5)	28 (66.7)		18 (42.9)	27 (64.3)		17 (40.5)	28 (66.7)	
Tumour site									
Parotid gland	32 (76.2)	34 (81.0)	0.595	30 (71.4)	36 (85.7)	0.111	32 (76.2)	34 (81.0)	0.595
Other	10 (23.8)	8 (19.0)		12 (28.6)	6 (14.3)		10 (23.8)	8 (19.0)	
T-stage									
T1-T2	20 (50.0)	20 (50.0)	1.000	21 (51.2)	19 (48.7)	0.823	20 (48.8)	20 (51.3)	0.823
T3-T4	20 (50.0)	20 (50.0)		20 (48.8)	20 (51.3)		21 (51.2)	19 (48.7)	
Missing information[b]	2	2		1	3		1	3	
N-stage									
N0	29 (69.0)	18 (42.9)	**0.016**	26 (61.9)	21 (50.0)	0.272	25 (59.5)	22 (52.4)	0.510
N1-N3	13 (31.0)	24 (57.1)		16 (38.1)	21 (50.0)		17 (40.5)	20 (47.6)	
Histological subtype[c]									
ACC	2 (6.1)	5 (13.9)	**0.005**	2 (5.7)	5 (14.7)	**0.018**	2 (5.9)	5 (14.3)	0.124
MEC	4 (12.1)	6 (16.7)		5 (14.3)	5 (14.7)		4 (11.8)	6 (17.1)	
AdCC	15 (45.5)	3 (8.3)		15 (42.9)	3 (8.8)		13 (38.2)	5 (14.3)	
AdenoCa	11 (33.3)	15 (41.7)		11 (31.4)	15 (44.1)		13 (38.2)	13 (37.1)	
SCC	1 (3.0)	7 (19.4)		2 (5.7)	6 (17.6)		2 (5.9)	6 (17.1)	
Other[b]	9	6		7	8		8	7	
Subtype characteristic									
Non-aggressive	7 (18.4)	11 (26.8)	0.373	9 (22.0)	9 (23.7)	0.854	7 (17.9)	11 (27.5)	0.312
Aggressive	31 (81.6)	30 (73.2)		32 (78.0)	29 (76.3)		32 (82.1)	29 (72.5)	
Non-classified[b]	4	1		1	4		3	2	

	CD8$^+$ invasive front			PD-L1			PD-1$^+$ total		
	Low **n = 42 (%)**	High **n = 42 (%)**	p-value[a]	TPS < 5% **n = 70 (%)**	TPS ≥ 5% **n = 14 (%)**	p-value[a]	Low **n = 42 (%)**	High **n = 42 (%)**	p-value[a]
Gender									
Male	27 (64.3)	24 (57.1)	0.503	42 (60.0)	9 (64.3)	0.764	27 (64.3)	24 (57.1)	0.503
Female	15 (35.7)	18 (42.9)		28 (40.0)	5 (35.7)		15 (35.7)	18 (42.9)	
Age (range, 9–93 years)									
< Median (61 years)	26 (61.9)	13 (31.0)	**0.004**	36 (51.4)	3 (21.4)	**0.040**	24 (57.1)	15 (35.7)	**0.049**
≥Median	16 (38.1)	29 (69.0)		34 (48.6)	11 (78.6)		18 (42.9)	27 (64.3)	
Tumour site									
Parotid gland	30 (71.4)	36 (85.7)	0.111	53 (75.7)	13 (92.9)	0.154	33 (78.6)	33 (78.6)	1.000
Other	12 (28.6)	6 (14.3)		17 (24.3)	1 (7.1)		9 (21.4)	9 (21.4)	
T-stage									
T1-T2	20 (50.0)	20 (50.0)	1.000	36 (54.5)	4 (28.6)	0.077	20 (51.3)	20 (48.8)	0.823
T3-T4	20 (50.0)	20 (50.0)		30 (45.5)	10 (71.4)		19 (48.7)	21 (51.2)	
Missing information[b]	2	2		4	0		3	1	
N-stage									
N0	27 (64.3)	20 (47.6)	0.124	43 (61.4)	4 (28.6)	**0.024**	23 (54.8)	24 (57.1)	0.826
N1-N3	15 (35.7)	22 (52.4)		27 (38.6)	10 (71.4)		19 (45.2)	18 (42.9)	

(*continued on next page*)

Table 3 (*continued*)

	$CD8^+$ invasive front			PD-L1			$PD\text{-}1^+$ total		
	Low **n = 42 (%)**	High **n = 42 (%)**	p-value[a]	TPS < 5% **n = 70 (%)**	TPS ≥ 5% **n = 14 (%)**	p-value[a]	Low **n = 42 (%)**	High **n = 42 (%)**	p-value[a]
Histological subtype[c]									
ACC	0 (0.0)	7 (20.0)	**0.002**	6 (10.7)	1 (7.7)	**0.046**	2 (6.1)	5 (13.9)	**0.033**
MEC	5 (14.7)	5 (14.3)		8 (14.3)	2 (15.4)		3 (9.1)	7 (19.4)	
AdCC	15 (44.1)	3 (8.6)		18 (32.1)	0 (0.0)		14 (42.4)	4 (11.1)	
AdenoCa	12 (35.3)	14 (40.0)		20 (35.7)	6 (46.2)		12 (36.4)	14 (38.9)	
SCC	2 (5.9)	6 (17.1)		4 (7.1)	4 (30.8)		2 (6.1)	6 (16.7)	
Other[b]	8	7		14	1		9	6	
Subtype characteristic									
Non-aggressive	5 (12.8)	13 (32.5)	**0.037**	16 (24.6)	2 (14.3)	0.403	8 (20.0)	10 (25.6)	0.550
Aggressive	34 (87.2)	27 (67.5)		49 (75.4)	12 (85.7)		32 (80.0)	29 (74.4)	
Non-classified[b]	3	2		5	0		2	3	

Abbreviations: ACC, acinic cell carcinoma; MEC, mucoepidermoid carcinoma; AdCC, adenoid cystic carcinoma; AdenoCa, adenocarcinoma – not otherwise specified; SCC, squamous cell carcinoma; TPS, tumour proportion score.

[a] Pearson chi-squared test.

[b] Not included into statistical analysis.

[c] Only the five most frequently diagnosed histopathological subtypes in the study cohort were considered.

on a total level ($p \leq 0.001$) and with respect to spatial distribution in the intratumoural compartment ($p = 0.002$), stroma ($p \leq 0.001$), and invasive front ($p = 0.029$). Additionally, a PD-L1 TPS ≥ 5% significantly coincided with a high amount of $PD\text{-}1^+$ TILs ($p = 0.003$) (Supplementary Table S2, Fig. 1, Supplementary Figs. S1 and S2), and the variables age and histological subtype (Table 3). There was no impact of $PD\text{-}1^+$ TILs on survival within the examined group (Table 2).

Discussion

To the best of our knowledge, this is the first study addressing the impact of both $CD8^+$ TILs and PD-L1 expression in salivary gland malignancies in terms of survival in a larger population and consideration of a semi-automated analysis method. With respect to our main results, patients with an elevated level of PD-L1 expression were significantly prone towards an impaired OS and PFS. Individuals with a high $CD8^+$ cell density especially in the invasive front, displayed a significantly decreased OS, and a negative trend regarding LPFS, DMFS and PFS.

Concerning PD-L1 expression, our findings are in line with previously published reports. Mukaigawa et al. examined a heterogeneous pool of 219 specimens and documented a significantly decreased OS and disease-free survival in patients exceeding a cut-off value of ≥1% PD-L1 expressing cells [15]. Harada et al. investigated a cohort of 47 specimens, and also observed a significantly decreased OS in patients exceeding a cut-off value of ≥5% PD-L1 expressing cells [19]. Sato et al. confirmed an impaired OS in patients with SDC and high PD-L1 expression (n = 18, cut-off value ≥ 10%) [20], whereas Mosconi et al. reported indifferent results in a population with MEC (n = 30, cut-off value ≥ 50%) [17]. Altogether, our results match with observations of high PD-L1 expression and unfavourable outcome in other tumours such as oesophageal cancer, gastric cancer, colorectal cancer, hepatocellular carcinoma, and urothelial cancer [36].

Although several studies reported a survival benefit associated with the presence of $CD8^+$ TILs for other entities [13,14], our observations in salivary gland cancer are in contradiction to that. This might be explained to a certain extent by exhaustion of these cells and their high prevalence only in the peritumoural stroma and invasive front, but not in the intratumoural compartment. In chronic infections and cancer, $CD8^+$ cells are continuously exposed to antigen and/or inflammatory signals [33]. On the long term, these cells acquire an exhausted and even dysfunctional state [37]: Mosconi et al. demonstrated in 36 specimens of AdCC high densities of $CD8^+$ TILs to coincide with low quantities of GrB expressing cells. Though in patients with a high density of $CD8^+$ cells a longer survival was observed [21], secretion of GrB demonstrates successful $CD8^+$ TILs activation and hence, effective anti-tumour immune response [38]. In conclusion, a high density of $CD8^+$ cells, but low quantities of GrB might demonstrate TILs exhaustion.

Besides, there are several cell populations of $CD8^+$ TILs of different functional status co-existing in tumour tissue, though it is unclear, whether these populations are randomly scattered, or if they are clustered in a distinct tumour compartment [37]. The latter theory might contribute to our findings, as individuals with a high prevalence of $CD8^+$ cells in the invasive front displayed an overall impaired survival. According to that thesis, particularly an abundance of $CD8^+$ TILs in a dysfunctional status might be clustered in the invasive front of head and neck salivary gland carcinomas, not being able to infiltrate the main tumour, which is also in line with the lower number of $CD8^+$ cells as observed in the intratumoural compartment.

Further, we were able to demonstrate a significant association of high counts of $CD8^+$ TILs and higher age, as well as with nodal involvement. These findings are in line with the concept of $CD8^+$ cell exhaustion, as in older patients with several co-morbidities a suppressed immune system seems likely, and high nodal stages often depict an advanced disease and thus, continuous exposition to antigen and/or

inflammatory signals.

The expression of cell surface inhibitory receptors such as PD-1, cytotoxic T-lymphocyte antigen 4 (CTLA4), lymphocyte activation gene 3 (LAG3), and T-cell immunoglobulin domain and mucin domain-containing protein 3 (TIM3) [39], or regulatory cells (T_{Reg} cells) such as forkhead box P3-expressing (FOXP3) CD4-positive cells [40,41], are possible indicators of TILs exhaustion [33]. As the PD-1 pathway displays a more specific effect on anti-tumour T-cells [13], and as it is still unclear whether T_{Reg} cells directly affect $CD8^+$ TILs exhaustion [33,41], we focused our investigation on PD-1 as an exhaustion marker. Here we were able to show relevant association for high expression of PD-1^+ with $CD8^+$ TILs, and hence, some evidence of $CD8^+$ cell exhaustion in our studied group.

With respect to the examined histopathological subtypes, particularly the immunohistochemical results concerning AdCC and SCC are of note. While in AdCC only a low expression of $CD8^+$ cells and a neglectable PD-L1 expression were observed, in SCC an elevated expression of $CD8^+$ TILs was found, although the results in terms of PD-L1 expression were indifferent. Regarding AdCC, Mosconi et al. and Chang et al. and reported similar results [16,21]. Based on a classification by Teng et al., aggressive salivary gland cancers as SCC represent Type I-tumours, demonstrating adaptive resistance, whereas others like AdCC are Type II-tumours, showing immunological ignorance [42]. This might be an indicator of an overall low immunogenicity in AdCC, and an unsuitable histopathological subgroup receiving targeted therapy. However, treatment by radiotherapy may enhance antigen-presentation, attract lymphocytes, and reverse these immunologically cold tumours into hot ones [43]. On the other hand, SCC of the salivary glands seems a promising entity for combined approaches including immunotherapy, as proposed for oropharyngeal SCC [11,44].

Emphasising the strengths of the present work, our investigation reports on one of the largest sample sizes examined thus far. The rarity of head and neck salivary gland tumours and the comparably long collecting periods justify representativeness of the heterogeneous sample [21]. To minimise selection bias, we evaluated total-scan slides instead of tissue microarrays. The latter might cause a sampling error, as distinct plugs, and thus small tumour portions out of a FFPE block are analysed [15,16]. Additionally, we used a digital image analysis approach to perform cell quantification, supporting objectivity and reproducibility [16,30].

Nevertheless, there are no standardised evaluation methods concerning the expression of CD8, PD-L1, and PD-1, by now. Individual cut-off levels vary between previous studies [15,16]. Based on a range of twenty histological subtypes and tumour heterogeneity [3], different treatment responses are expectable, complicating a general statement. Due to the rare prevalence of head and neck salivary gland neoplasms [1], prospective, randomised controlled clinical trials are challenging, why long-term retrospective studies are advised [45,46].

Conclusion

Immunological markers of interest should be examined in a broader context, as separate investigations might cause misleading interpretations. High numbers of exhausted $CD8^+$ TILs together with high PD-L1 expression in the sense of adaptive resistance as well as an immunological ignorance are distinct mechanisms of resistance found in salivary gland cancer. These factors and various histopathological entities require different therapeutic approaches.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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